LESSON 29:
METHODS OF DIRECT GENE TRANSFER

Introduction

The first transgenic plant was produced via Agrobacterium mediated modified transformation of Nicotiana tabacum protoplasts by Horsch and co-workers in 1984. Since then several dozen plant species have been genetically engineered using different techniques. Simultaneous development of other techniques such as selectable markers facilitated the development in genetic engineering for obtaining transformed plants. But this technique is not suitable for monocotyledon plants as they are not natural host of Agrobacterium (there is evidence that limited gene transfer is possible in monocots by this system). Therefore, other methods of direct gene transfer have been developed for use with monocots and other species. These can be categorized.on the basis of the use of protoplasts or cell and tissue as the target materials. The isolation and purification of protolasts have been presented in earlier Lessons. Freshly isolated protoplasts are used for genetic transformation using direct gene transfer methods.

Let us understand the procedures of DNA transfer in Protoplasts and Plant tissues in detail:

A. DNA Transfer in Protoplasts

This is accomplished by:

i. Electroporation .

ii. Chemically stimulated DNA uptake by protoplasts

iii. Liposomes

iv. Microinjection

v. Sonication

B. DNA Transfer in Plant Tissues

i. Acceleration of DNA coated microparticles

ii. Laser microbeam

iii. Silicon carbide fibres

Direct uptake of DNA by isolated protoplasts is a genotype dependent response. Regeneration from protoplasts is not common in all the species. Due to this, production of fertile transgenic plants has remained difficult in most of the cereal species. However, transgenic fertile plants have been produced in Sorghum vulgare, Oryza sativa and Hordeum vulgare. Direct delivery of free DNA molecules into plant protoplasts by physical (electroporation and micro injection) and chemical (polyethylene glycol) methods have been developed to facilitate DNA delivery across the plasma membrane.

Dna Transfer in Protoplasts

A. Electroporation

This method is based on the use of the short electrical pulses of high field strength. Electroporation causes the uptake of DNA into protoplasts by temporary permeabilization of the plasma membrane to macromolecules. Protoplasts and foreign DNA are placed in a buffer between two electrodes and a high intensity electric current is passed (Fig.1.). Electric field damages membranes and creates pores in membranes. DNA diffuses through these pores immediately after

electric field is applied, until the pore are resealed. The technique is optimized by using appropriate electric field strength (defined as the applied voltage divided by the distance between two electrodes). The optimum field strength is dependent on the following:

1. The pulse length of electric current
2. Composition and temperature of the buffer solution
3. Concentration of foreign DNA in the suspension
4. Protoplasts density, and
5. Size of the protoplasts.

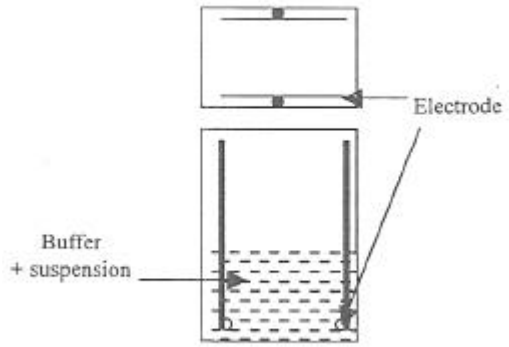

Fig.1. Top (above) and side view (below) of glass cell with electrodes used for electroporation.

It has been demonstrated that the removal of pectin from the plant wall increases the amount of DNAwhich can be introduced by electroporation. Tobacco mosaic virus was introduced in tobacco protoplasts by this method. Electroporation has been used successfully for transient and stable transformation of protoplasts from a wide range of species. Plating efficiency (i.e. number of colonies recovered out of number of cells transferred on plates) of electroporated protoplasts grown on selection medium (containing selective marker) can be as high as 0.5%. The highest plant transformation efficiencies have been reported for tobacco, with 0.2% of electroporated leaf mesophyll protoplasts giving rise to transgenic calli. Low transformation efficiency is common in cereals, e.g. in rice 0.002% efficiency was recorded.

B. Chemically Stimulated DNA Uptake

Direct uptake of DNA by protoplasts is stimulated by polyethylene glycol (PEG) and PEG is the most widely used chemical for this purpose. PEG mediated transformation involves mixing of freshly isolated protoplasts with DNA and immediately adding 15-20% PEG dissolved in a buffer containing

divalent cations. This mixture is incubated for 30 minutes, protoplasts are washed and then plated in petri plates for culture and growth. The optimization of transformation frequencies by this method include factors that follows.

1. PEG concentration in the mixture
2. Composition and concentration of salts used
3. The pH of the solution
4. Concentration of the foreign DNA
5. Size and form (linear, supercoiled) of the DNA molecules used
6. Culture and selection techniques used for protoplasts.

PEG mediated transformation is generally preferred over electroporation for stable transformation of monocot protoplasts due to relatively higher survival rates after treatment. PEG also stimulates the uptake of liposomes and improves the efficiency of electroporation. PEG causes precipitation of ionic macromolecules like DNA and stimulates their uptake by endocytosis. PEG mediated DNA uptake typically transforms 0.1 to 0.4% of the total protoplasts treated. Production of transgenic plants depends upon the regeneration competance of the transformed protoplasts. In case of Petunia, 40% transformed calli derived from mesophyll protoplasts could be induced to form fertile plants. This is equal to about 0.1% transformation efficiency of the treated protoplasts. In different species different transformation efficiency has been observed, e.g., in embryogenic protoplasts suspension of rice was 0.0004% and, soyabean and tobacco was 0.7-1%.

C. Liposomes

Liposomes have also been used as a carrier for the introduction of nucleic acid into plant protoplasts. Liposomes are small lipid sacs containing plasmids and are prepared artificially. The fusion of liposomes with plant protoplasts is stimulated by chemicals such as PEG (endocytosis). Liposomes mediated transformation has been achieved by including positively charged agents such as cations in the transformation mixture or using the cationic liposome preparation (Fig.2.).

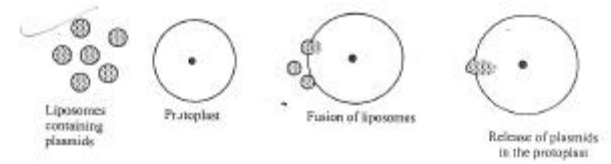

Fig. 2. Liposomes mediated gene transfer.

Other chemical agents like polycation Polybrene or lipofectin have also been used for both transient and stable transformation for maize protoplast. Cationic liposome and polycation mediated DNA delivery are new protoplasts transformation methods and are considered better than other methods of transformation. There are several advantages for the use of this technique.

1. Protection of DNA/RNA from nuclease digestion.
2. They have low cell toxicity.
3. Encapsulation of nucleic acids makes them more stable during storage.
4. High degree of reproducibility.
5. This method is applicable to wide range of plant cell types.

D. Microinjection

Delivery of nucleic acids to protoplasts or intact cells via microinjection is a labour intensive procedure that requires special capillary needles, pumps, micromanipulators, inverted microscope and other equipment. However, injection into the nucleus or cytoplasm is possible and cells can be cultured individually to produce callus or plants. In this way selection of transformants by drug resistance or marker genes may be avoided. This method involves skill of the worker to insert needle into the cytoplasm or in the nucleus. The basic technique is similar to that used for animal cell microinjection. In order to microinject protoplasts or other plant cells, the cells need to be immobilized (Fig.3). The cells are immobilized by:

1. The use of a holding pipette which holds the cells by vacuum.
2. Attachment of cells to poly-L-lysine coated cover slips.
3. Embedding the cells in agarose, agar or sodium alginate.

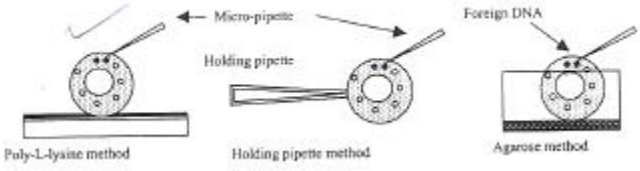

Fig. 3. Various methods of immobilizing the cell and microinjection

Glass micropipette are prepared to have openings of about 0.3 uM in diameter and are inserted into plant cell cytoplasm and nuclei with the aid of a micro manipulators device. A syringe like device is used for the controlled delivery of volume (10-11 - 10-4 ul) into the plant cell. Most plant cells are injected while keeping inside microdroplets (2-50 ul) of medium using a chamber which is sterile, vibration free and permits temperature and humidity regulation. A maximum of 100-200 cells per hour can be microinjected by this method.

The recovery of transformants is dependent upon the regeneration ability of the microinjected cells. Different methods have been used to grow injured (microinjected) single cells or protoplasts. Hanging droplets, covered under thin layer of agar or agarose, and micro culture have been used (Fig.4). Attempts have been made to inject linear, or super coiled DNA, in cytoplasm or in nucleus. Nuclear injections are found better for transformations.

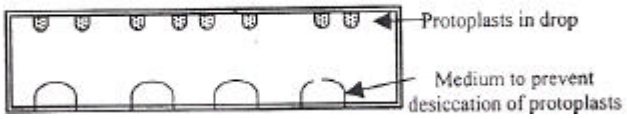

Fig. 4. Hanging Drop culture method

E. Sonication

Mild sonication (20 KHz ultra-sound) has been used to facilitate the uptake and transient expression of a chloramphenicol acetyltransferase (CAT) gene in protoplasts of sugar beet

(Beta vulgaris) and tobacco. This method was found superior than electroporation method used for the same material. Plating efficiency was also similar to untreated cells. However, transgenic plant production using this technique has not been reported so far.

Free DNA Transfer to Intact Tissue

A. Acceleration of DNA Loaded Microparticles (Particle Gun or Biolistic Method)

This is latest technology to transfer DNA into intact tissues. Several devices are developed using different methods. All to achieve the transfer of micro-sized particles (microprojectiles) coated with DNA to penetrate the cells. In this procedure micron size tungsten or gold particles are accelerated in a gun barrel to velocities sufficient for non-lethal penetration of cell walls and membranes. Klein and co-workers in 1987 developed and used for the first time the particle gun to transfer chimeric DNA and viral RNA molecules into intact onion cells. Tungsten acted as a carrier of nucleic acids because it was available in micro-size balls, non-toxic to cells and dense enough (high density) for rapid penetration of target material.

Microprojectile mediated transformation is a mechanical method of introducing DNA in to any plant species. This method can be successfully used where plasmids or protoplasts mediated transformation cannot be used. An acceleration device used to propel particles (micro projectiles) carrying plasmid DNA is called by various names based on machine or technique used to accelerate the particles such as 'particle gun technology', 'biolistic method', 'DNA bombardment', 'particle acceleration of DNA method' and 'electric discharge particle acceleration method'.

This is a quick method of stable transformation and testing a gene for cell and organelle specific expression. This technique has three components -

1. The basic equipment to generate particle acceleration.
2. Metal particles coated with precipitated DNA (desired gene).
3. Plant tissues to be used for particle penetration. The method for regeneration should be previously standardized and proper tissues be selected for bombardment.

(a) Instrument: The instrument is commercially available. Prototype was designed by Klein and co-workers. It uses the explosive force of gun powder (0.22 caliber gun cartridge) to accelerate a polypropylene cylindrical macroprojectile. Thin piece of polypropylene macroprojectile is loaded with microprojectiles coated with DNA. Gun powder explosion forces this macroprojectile to move with high speed toward another end of barrel, where it is blocked by a polycarbonate disc having an aperture. Macroprojectile is stopped but microprojectiles move fast through the aperture towards tissue placed in the same direction. For each transfer, 50 mg tungsten is accelerated upto 2000 ft per second in a partial vacuum. With this speed, particles reach upto lower layers of cells in target tissues (Fig.5).

The other devices are similar in basic design concept but use different methods to accelerate particles like use of compressed air or gas. Compressed air (130 kg/cm2 pressure) has been used to accelerate microprojectiles at velocities (approximately 440 m/sec) necessary to achieve DNA delivery to plant cells (Fig.6).

An electric discharge particle acceleration device differs in basic design from the above described devices. In this device, a high voltage discharge (14 KV current) delivered to a small water droplet which quickly vaporizes and releases energy to propel DNA coated gold spheres into target cells (Fig.7). In a similar way to above devices, a DNA carrier is attracted (accelerated) due to potential differences, stopped in-between by a screen, DNA coated particles cross the screen and fly towards target tissue and deliver the DNA into cells

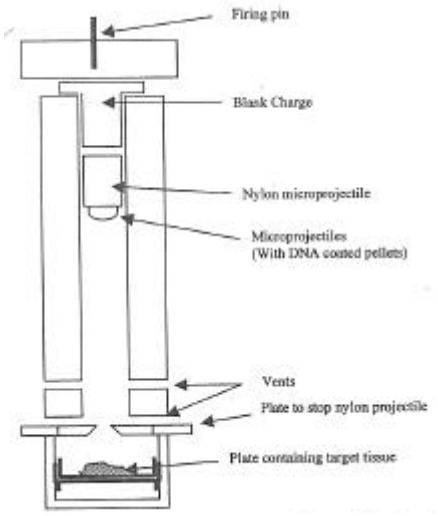

Fig. 5. Particle gun or shotgun for delivering DNA coated microprojectiles into plant cells

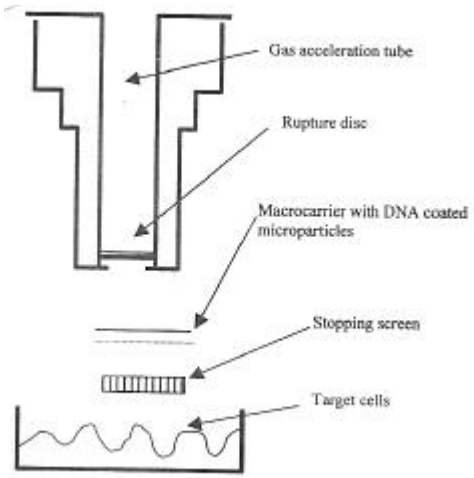

Fig.6. Compressed air particle acceleration device for delivering DNA coated microprojectiles into plant cells

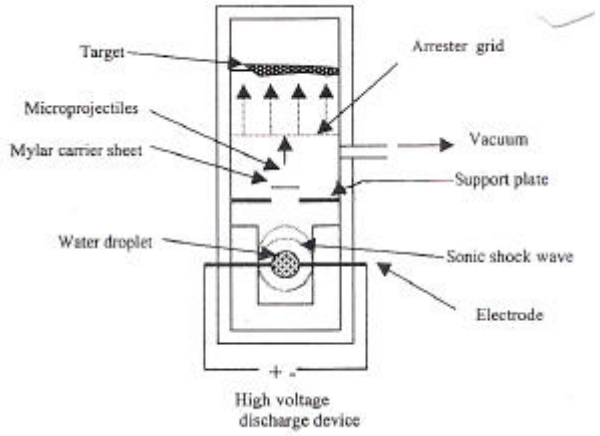

Fig. 7. Particle acceleration device based on high voltage charge for delivering DNA coated microprojectiles into plant cells.

b DNA coating

This is a sophisticated technology and requires precise preparation of DNA coated gold or tungsten particles. The particles should have following properties:

1. High density (19 g/cm^3 or greater) to ensure proper acceleration and penetration through cell walls.
2. Size (0.5-5 um) should match with size of the cells. Large sized spheres can be used with large cell size.
3. Gold is costlier than tungsten, but it does not oxidize like tungsten.

DNA is precipitated onto the particles prior to bombardment. Most commonly, CaCl2 and spermidine are used to precipitate plasmid DNA (the desired gene attached to plasmid is suitable for integration into plant genome) onto tungsten particles. Ethanol is also used for precipitation of the DNA on gold particles.

c. Plant tissue

Plant tissue used for transformation should be competent to regenerate. Mostly embryogenic tissue is an ideal material for transformation (e.g., in corn, cotton, soyabean, papaya and wheat) but transformed plants have been obtained after leaf bombardment in tobacco, and stem bombardment in cranberry. Normally, reporter gene and selectable marker genes are used to isolate and select transformed cells/plantlets. Transgenic plants produced by this technique are listed in Table 1.

Table 1. Transgenic plants formed by microprojectile bombardment of various plant cells

Plant species	Cell source
Corn	Embryonic cell suspension, immature zygotic embryos (IZE)
Rice	IZE, embryogenic callus
Barley	IZE, cell suspension,
Wheat	IZE,
Sorghum	IZE
Pearl millet	IZE
Orchid	Protocorms
Banana	Embryonic cell suspension
Pea	Zygotic embryos
Cucumber	Embryogenic callus
Cotton	Zygotic embryos
Grape	Embryonic cell suspension
Tobacco	Pollen

B. Laser Microbeam

Weber and co-workers (1988) demonstrated use of laser beam for transformation of plant cells. An ultraviolet (UV) laser microbeam has been used to introduce DNA into plant cells and chloroplasts. A 343 nm beam (wavelength of UV is 200 to 400 nm) is directed through an adjustable attenuator into the optical path of an inverted microscope. The focus of the laser beam is adjusted so that it is identical with that of the objective lens. The laser beam is targeted by focusing on a specimen in the microscope. This laser beam can then make holes in any part of cell which is in focus. Laser micropuncture of the cell wall and plasma membrane allows uptake (entry) of plasmid DNA into cells. Brassica napus (rape seed) cells and microspores have been used for transformation by this technique. This technique has also been used to transfer genes into isolated chloroplasts and chloroplasts of intact protoplasts. 20% transformation was achieved by this method but fertile plants are yet to be produced by this method.

C. Silicon Carbide Fibres

Microinjection and electroporation methods have also been used for transfer of DNA using intact plant cells and tissues. Similarly, vortexing plasmid DNA and plant cells with silicon carbide fibre (0.6um in diameter and 10-80 um in length) produced transformed cells at low frequency. Under vortex (vigorous shaking by vibration), silicon fibres penetrate cells and create fine holes permitting entry of DNA.

DNA uptake by imbibition in dried embryos of cereals and legume species has also been reported. This is a very simple method and dried somatic embryos can also be used for this method. Only transient gene expression has been observed and stable transformations are yet to be achieved by this method. Attempts have been made to introduce gene in pollen grains by microinjection in the anthers. These pollen grains can be used for fertilization to obtain transgenic plants.

Examples of Transgenic Plants

Herbaceous Dicot - Tobacco, Petunia hybrida, tomato, potato, eggplant, Arabidopsis thaliana, lettuce, Apium graveolens (celery), sunflower, Flax, rape oil seed, cauliflower, cabbage, cotton, soyabean, pea, chicory, liquorice, sweet potato, kiwi, papaya, grape, rose, Chrysanthemum, etc.

Woody Dicot - Populus, Malus, Pyrus communus, Azadirachta indica

Monocot - Asparagus, Secale cereale, Oryza sativa, Triticum aestivum, Zea mays, Avena sativa

Gymnosperm - Picea glauca.

Conclusion

Recombinant DNA technology has made the transfer of genes from any organism to any other organism a technical feasibility. Although, Agrobacterium mediated gene transfer has been the most common method for gene transfer in most of the dicot plants, its application to monocots is limited, since this does not infect monocots. For this reason, several methods of direct gene transfer are employed for obtaining transformation in plants. This Lesson gives a brief account of the various methods of direct gene transfer which are useful in producing transgenic plants.

Questions

1. Describe the various methods of direct gene transfer giving suitable examples.
2. Write short notes on the following:
   a. Transgenic plants
   b. Particle acceleration
   c. DNA uptake by protoplasts
   d. Transgenics for disease resistance
   e. Microinjection
   f. Electroporation
   g. Lasers and Liposomes for transformations.
3. Give a few examples of transgenic plants.

Note